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Prevalence of Shiga toxin – producing *Escherichia coli* in food products of animal origin as determined by molecular methods

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Running title: STEC prevalence in food

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Abstract

In this study we report on the prevalence and distribution of Shiga toxin-producing *Escherichia coli* (STEC) in food products of animal origin, collected in the Piedmont region of Italy, as determined by a combination of quantitative PCR (qPCR) protocols, applied directly to the samples, and of culture-dependent isolation and subsequent molecular identification and characterization of isolates. The qPCR protocols were developed and optimized in this study and targeted the *rpoB* gene (as a marker for total *E. coli*) and the stx_1, stx_2 and *eaeA* genes (as markers for potentially virulent *E.coli*). They were then used to test for STEC in 101 food samples, before and after enrichment. A STEC prevalence of 42 % (21/50) for dairy products and 70 % (36/51) for meat products was obtained. Fifty four isolates were identified as STEC and led to a culture based prevalence of 36 % and 27.4 % for dairy and meat products, respectively. A large number of strains carried the stx_2 gene (39 out of the 54 STEC strains) compared to strains that carried stx_1 (30 out of 54), only a limited number of them contained the *eaeA* gene (11 out of 54) while 14 strains contained both stx_1 and stx_2. Eight of the 54 isolates belonged to the O157 serogroup, and non belonged to serogroups O26, O145, O111 or O103. Strains isolated from meat products were diverse, as determined by Enterobacterial repetitive intergenic consensus-PCR (ERIC), while those isolated from dairy products were more similar and grouped together by cluster analysis. The results of the culture-independent approach showed a high prevalence of STEC in dairy and meat based products, mainly fermented, indicating a possible safety risk for these types of foods commodities.

Key-words: STEC, qPCR, prevalence, distribution in food

1. Introduction

The distinguishing feature of Shiga toxin-producing *Escherichia coli* (STEC) from other pathogenic *E. coli* is the production of one or more Shiga toxins (Stx1 and/or Stx2) (Paton and Paton, 1998a). STEC comprise a diverse group of microorganisms, encompassing strains of *E. coli* with different virulence genotypes, serotypes, epidemiology and disease causing potential. This diversity is highlighted by the classification of STEC into five seropathotype groups, based on the frequency of association of certain serotypes with serious and epidemic human disease (Karmali et al., 2003). *E. coli* O157:H7 is the serotype which has caused most foodborne outbreaks and has led to the highest number of hemolytic uremic syndrome (HUS) cases, a life-threatening sequela of STEC infection. However, there is increasing evidence that other serotypes can also cause severe human disease (Caprioli et al., 1997, 2005; Paton and Paton, 1998a).

Research regarding STEC has so far mainly focused on isolates from patients who have suffered a STEC infection. Information regarding the prevalence and distribution of STEC in foodstuffs is only recently becoming available. Studies conducted so far (Brooks et al., 2001; Doyle and Schoeni, 1987; Fantelli and Stephan, 2001; Pradel et al., 2000), particularly those investigating STEC strains with different virulence genotypes (Pradel et al., 2008; Slanec et al., 2009), highlight the need for determination of a possible association of certain STEC genotype profiles with specific food types in order to assess their possible risk to human health.

In order to complement the information regarding prevalence of pathogens in food which is acquired by conventional sampling and testing, molecular methods that can be applied directly in the food, without prior culturing, are being increasingly used. It

has become evident that the information regarding presence and viability of pathogens obtained by culture-independent approaches is, in many cases, different from the information gathered by culture-dependent approaches (Auvray et al., 2009; Madic et al., 2011; Pradel et al., 2000). Furthermore, in a 'farm-to-fork' study, taking into consideration the prevalence of STEC throughout the lamb food chain, it was concluded that qPCR detected virulent populations in sampling points (environmental and meat samples) where conventinal microbiological methods failed (Oses et al., 2010).

In this study we report the optimization of a qPCR approach for the detection and quantification of total *E. coli* and STEC sub-populations in foodstuffs. The optimized protocols, targeting the *rpoB* and 3 virulence genes (stx_1, stx_2 and *eaeA*), were applied on 101 food samples of animal origin in order to detect total *E.coli* and determine the prevalence of STEC, respectively. Furthermore, a culture-based method was compared to the qPCR method for STEC detection, and the virulence genotypes of the isolated STEC from dairy and meat products were determined.

2. Materials and methods

2.1 Sampling

One hundred and one traditional products of animal origin, produced in the Piedmont region of Italy, were analyzed for the presence of STEC. Fifty of them were dairy products, produced from un-pasteurized milk and fifty one were meat products, both fresh and fermented (supplementary Table 1). For sampling and enrichment, 10 g of each product were mixed with 40 ml of Brain Heart Infusion broth (BHI, Oxoid, Milan, Italy), and homogenized for 2 minutes in a stomacher machine (Interscience, Turin, Italy). One ml of a 1 to 10 dilution of the homogenate in Ringer's solution was

immediately centrifuged for 5 minutes at maximum speed and after removal of the supernatant, the pellet was stored at –20 ºC for DNA extraction at a later stage (sample at t=0). The homogenate and appropriate serial decimal dilutions were also plated on Sorbitol McConkey Agar (SMAC, Oxoid) and incubated at 37 ºC for 24 hours. Then, the homogenate was incubated at 37 ºC for 24 hours. After that, 1 ml of a 1 to 10 dilution of the enriched homogenate was centrifuged as above and the pellet obtained was stored at –20 ºC until the DNA extraction (sample at t=24 h). Similarly, a loopfull of the enriched homogenate was streaked on SMAC medium and incubated at 37 ºC for 24 hours. Whenever possible, 10 colonies from the SMAC plates, both pink and straw –colored, were randomly selected for each sample (5 at t=0 and 5 at t=24 h), streaked for isolation and subjected to DNA extraction and PCR for molecular identification and characterization, as described below.

2.2 qPCR protocols and construction of calibration curves

For the qPCR amplification and the determination of the STEC population(s) in food samples, four genes were targeted: *rpoB*, encoding the β-subunit of the RNA polymerase (used for detection/quantification of total *E.coli* populations), stx_1, encoding the Shiga toxin type 1, stx_2, encoding the Shiga toxin type 2 and *eaeA*, encoding the intimin outer membrane protein. The sequences of primers and probes developed in this study as well as the amplification conditions are listed in Table 1. The amplification for the *rpoB* gene is specific for the *E. coli* species while amplifications for stx_1, stx_2 and *eaeA* are specific for STEC. The specificity of the primers and probes was tested using DNA from 100 strains, belonging to 50 different species. Pathogenic and spoilage microorganisms (with particular emphasis on enterobacteria) as well as technologically important microorganisms (mainly lactic acid bacteria and coagulase negative staphylococci), were considered. All strains

belonged to the culture collection of the University of Turin and comprised international culture collection strains and strains isolated from foods and previously identified by sequencing of partial 16S rRNA gene (Cocolin et al., 2000, 2002; Rantsiou et al., 2006, 2008) . The amplification, for all four genes, was carried out in 25 µl final volume, containing 12.5 µl of the 2X FluoCycle mix for probe (Euroclone, Milan, Italy), 1 µl of 10 µM of each of the primers, 0.625 µl of 10 µM of the probe and 1 µl of DNA. The PCR conditions (Table 1) were optimized to generate *E. coli* species-specific amplification (for the *rpoB* gene) or for STEC (for the stx_1, stx_2 and *eaeA* genes). For the construction of the calibration curves, *E. coli* ATCC 35150 (encoding stx_1, stx_2 and *eaeA* genes) was artificially inoculated into different food matrices. Ten g were contaminated with serial dilutions of the *E. coli* strain to reach final concentrations that ranged from 10 to 10^8 colony forming units (cfu)/g. Then, 40 ml of BHI were added in the samples, which were then homogenized in a stomacher. One ml of the homogenate was diluted in 9 ml of Ringer's solution and 1 ml samples of the diluted homogenate were collected for later DNA extraction and amplification. The remaining homogenate was incubated at 37 ˚C for 24 hours and the dilution, DNA extraction and amplification were performed also from these samples. For each gene and each type of food matrix, the calibration curves were constructed by plotting the C(t) value obtained from the qPCR against the microorganism counts (in cfu/g). The efficiency of the reactions was calculated according to Rutledge and Côte (2003). Experiments were repeated three times.

2.3 Extraction of DNA from food matrices

DNA was extracted from the pellets obtained after the sampling, using the Master-Pure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA) following the manufacturer's instructions.

2.4 DNA extraction from pure cultures of isolates

For isolates grown in pure culture, DNA was extracted as previously described by Rantsiou et al. (2006). After extraction, the DNA was quantified and standardised to 100 ng/µl concentration.

2.5 Identification of STEC food isolates and serogroup determination

Isolates, were identified as STEC by testing for the presence of the *stx*$_1$, *stx*$_2$, *eaeA* and *hly* genes by multiplex PCR amplification, as described by Paton and Paton (1998b). To determine if isolates belonged to serogroups O26, O103, O111, O145 and O157, a 5'-nuclease assay was performed according to Perelle et al. (2004).

2.6 Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR

The ERIC-PCR was performed on the STEC isolates according to Versalovic et al. (1991) with a modification of the amplification cycle as follows: 30 cycles of denaturation at 90 °C for 30 seconds, annealing at 40 °C for 1 minute and extension at 65 °C for 8 minutes, preceded by an initial denaturation at 95 °C for 5 minutes and followed by a final extension at 65 °C for 15 minutes. The amplification products were analyzed by agarose gel electrophoresis (1.5% w/vol) in TAE 1X for 4 h at 120 V. After the run, gels were stained in TAE 1X containing 0.5 µg/ml ethidium bromide for 30 min and then observed under UV illumination by using the UVI pro Platinum 1.1 Gel Software (Eppendorg, Hamburg, Germany) for the recognition of the bands present. Comparisons of the fingerprints obtained for the STEC isolates were performed using the pattern analysis software package, Bionumerics, Version 4.6 (Applied Maths, Kortrijk, Belgium). Calculation of similarity in the profiles of bands was based on Pearson product– moment correlation coefficient. Dendrograms were obtained by means of the Unweighted Pair Group Method using Arithmetic Average

(UPGMA) clustering algorithm (Vauterin and Vauterin, 1992). Isolates were subjected twice to ERIC amplification.

3. Results

3.1 Method optimization and calibration curves

For the application of the proposed qPCR protocols in real food samples, the specificity of the employed primers/probes was tested. The conditions reported in the materials and methods section were chosen as specific for the entire *E. coli* species (*rpoB* gene) and the STEC subgroup (*stx* and *eaeA* genes) (data not shown). In order to be able to quantify possible STEC populations in naturally contaminated food samples, appropriate calibration curves were constructed. Briefly, this was done by artificially inoculating serial dilutions of a STEC strain in 2 different food matrices, cheese and fresh meat. Then the DNA was extracted and used as target in qPCR. For all the genes tested and in both matrices, a quantification limit in the order of 10^2 cfu/g was obtained. The linearity range spanned from 10^2 to 10^6 cfu/g. After an overnight enrichment in BHI it was possible to detect also 1-10 cfu/g (data not shown). The amplification efficiency and correlation coefficient (R^2) for each gene are reported in Table 2. Regarding the amplification efficiencies, comparable results were obtained for the *rpoB*, stx_1 and stx_2 genes with values ranging from 84.7 (for the stx_2 gene in fresh meat) to 97.2 (for the stx_1 gene in soft cheese). A lower efficiency was observed in the case of the *eaeA* gene. Some variation was observed in the R^2: for the *rpoB* gene the values obtained were over 0.990, while for the *stx* genes ranged from 0.970 to 0.988. Also in this case, slightly lower values were obtained for the *eaeA* gene.

3.2 Prevalence of total E. coli and STEC determined without culturing

The optimized qPCR protocols were employed in order to determine the prevalence of total *E. coli* and STEC in food samples. Total DNA was extracted from the

homogenate at t=0 and after 24 hours of enrichment. The results obtained for the four genes considered in this study are summarized in Table 3. A high percentage of samples resulted positive for the presence of total *E. coli* (83.2 %). Regarding the virulence genes targeted, a relatively higher percent of samples resulted positive for the stx_1 gene (about 54 %), with the stx_2 coming second (about 14 %) and lastly the *eaeA* gene was only present in 12 % of the samples at t=24h. With the exception of the *eaeA*, for which a difference in the percent of positive samples was observed between t=0 and t=24h, for stx_1 and stx_2, the number of positive samples did not vary between the two sampling points. It can be clearly seen that a higher number of meat products carried the stx_1 gene (over 70 %), while only a limited number of samples were positive for stx_2 (5.9 %) and *eaeA* (11.8%). On the other hand, dairy products presented a more balanced distribution of the stx_1 and stx_2 genes, even if, also in this case, stx_1 was more frequently detected. Also in the case of dairy products, *eaeA* was detected in a limited number of samples. For 6 dairy samples and 1 meat sample, it was possible to quantify the STEC population(s) based on the calibration curves constructed. For the rest of the samples, the presence of STEC populations was determined to be at or below the quantification limit. For the quantified samples, 6 (5 dairy and 1 meat sample) out 7 contained the stx_1 and stx_2 in loads that ranged from 10^2 to 10^4 cfu/g while one dairy sample contained all three virulence genes in loads of 10^3 cfu/g (Table 4).

3.3 Virulence genotyping of STEC isolates

Colonies were randomly selected from the SMAC plates at t=0 and at t=24h and were subjected to multiplex PCR amplification in order to identify those that were STEC. A total of 250 isolates were screened and 54 resulted to be STEC (possessing stx_1, stx_2 or both genes). The specific genotypes of the strains, with respect to the virulence

genes tested, namely *stx*$_1$, *stx*$_2$, *eaeA* and *hly*, and the source of isolation are shown in Table 5. For a total of 32 samples (31.7 %) it was possible to isolate and identify at least one STEC strain. Dairy products were slightly more contaminated (18 out of 50) with respect to meat products (14 out of 51). Strains carrying the *stx*$_1$ gene were isolated from 19 of the 101 samples (18.8 %) while the *stx*$_2$ gene was present in isolates from 28 of 101 samples (27.7 %) and the *eaeA* gene in isolates from 10 of 101 samples (9.9 %). As can be seen from Table 5, for 9 of the 18 positive dairy samples and for 7 of the 14 positive meat samples, a mixed STEC population was detected (since strains with different genotypes were isolated from the same sample). Lastly, the total number of strains isolated at t=0 was higher than at t=24h. Interestingly, for 9 samples, strains with different virulence gene profile were isolated before and after enrichment.

Eight STEC strains were determined to belong to the O157 serogroup while none of other serogroups tested (O26, O103, O111 and O145) was detected among the strains. Two of the O157 strains were isolated from two dairy samples while the remaining 6 were isolated from 4 different meat products. Seven out of the eight strains were isolated from samples at t=0 and 1 at t=24.

3.4 Molecular characterization of STEC strains

Strains identified as STEC were molecularly characterized by ERIC PCR. The fingerprint profiles obtained after agarose gel electrophoresis of the PCR products were statistically evaluated by cluster analysis and a dendrogram of similarity was constructed (Figure 1). Using a coefficient of similarity of 70 %, 9 clusters, containing at least 2 strains, were obtained, together with many strains positioned independently within the dendrogram. The most numerous were clusters 1, 2, 4, and 7 with 5, 5, 4 and 13 strains, respectively. It is worth noticing that these clusters primarily contained

strains isolated from dairy samples, for example for cluster 1, all 5 strains were of dairy origin while for cluster 7, 12 strains were of dairy origin and only one of meat origin. In many cases, strains isolated from the same sample grouped together, e.g. strains from sample 35F grouped in cluster 4, strains from sample 14F in cluster 7 and strains from sample 40F in cluster 2. Similarly, some strains isolated from meat samples grouped in the same cluster, e.g. strains of sample 39SGL in cluster 8, strains from sample 1SGL in cluster 2 and strains of sample 8SGL in cluster 9. Furthermore, a correlation of the clustering with the virulence genotype of the contained strains could be observed in many cases. Clusters 1 and 4 consisted mainly of strains carrying the stx_2 gene, clusters 3 and 9 were composed of strains encoding both stx_1 and stx_2, while the latter cluster also grouped together two strains of the O157 serogroup. The remaining isolates, 9 from meat and 7 from dairy products, were positioned in the dendrogram as single strains.

4. Discussion

In this paper a comprehensive study is presented that aimed at determining the prevalence as well as distribution of STEC populations in traditional food products of animal origin. For this purpose, qPCR protocols targeting the *rpoB* (for the total *E. coli* populations) and the stx_1, stx_2 and *eaeA* (for the STEC sub-populations) genes, allowing a quantification of the target microorganism, were designed and optimized. In parallel, conventional, culture dependent sampling was performed, with the goal of isolating STEC strains to study their virulence genotype and for characterization by PCR-based methods.

The first step was the optimization of the qPCR protocols with the goal of applying such protocols ideally without an enrichment, so that the analysis time is shortened

and a real quantification, without the need for plating, is possible. This, to a certain extent, was achieved since a good quantification limit was obtained: 10^2 cfu/g, in the two food matrices tested and for all genes targeted. However, for STEC populations below 10^2 cfu/g, an enrichment step was deemed necessary. Recently, it was proposed that it is preferred to utilize a non-selective enrichment medium for *E. coli* O157:H7. This allows resuscitation of sublethally injured cells without risking outgrowth by competing flora since *E. coli* is a fast grower (Jasson et al., 2009). In this study, we employed an overninght enrichment in BHI, which gave the possibility to detect also 1-10 cfu/g of sample. An advantage of performing an enrichment step is associated with the fact that while at t=0 the signal obtained in the qPCR could be attributed to DNA originating from dead cells, at t=24h the signal obtained is necessarily associated with alive and multiplying populations (Rossen et al., 1991). With the optimized protocols, calibration curves were obtained with good linearity range, efficiency of amplification and coefficient of similarity. The values obtained for these parameters in this study are comparable to those of other studies, dealing with detection and quantification of pathogens in foodstuffs (Rantsiou et al., 2008, 2010). Quantification was possible for 7 contaminated samples that contained STEC loads ranging from 10^2 to 10^4 cfu/g.

In approximately 83 % of samples, *E. coli* populations were detected, with dairy products presenting lower level of contamination (74 %) with respect to meat products (92 %). This trend was also seen when the STEC populations were taken into consideration. More specifically, regarding the dairy samples, 42 % resulted to contain one or more STEC populations while for the meat products this percentage was about 70%. It should be noted that limited differences were observed in these values between t=0 and t=24h, meaning that the majority of the samples contained

live cells. The virulence gene amplified with the highest frequency was the *stx*$_1$ (about 54 % of total samples), followed by the *stx*$_2$ (about 14 %) and the *eaeA* (about 12 %). This difference was particularly evident in the meat products, while for the dairy products, a relatively high percent of samples was also positive for the *stx*$_2$ gene. It can be said that overall, the positive meat products were characterized by *stx*$_1$ presence while in dairy products both *stx*$_1$ and *stx*$_2$ genes were of relevance. While for the samples positive for the *stx*$_1$ and *stx*$_2$ genes, no difference was observed between the t=0 and the t=24h, for the *eaeA* an increase in the number of positive samples could be seen after the enrichment. This result most likely means that population(s) carrying the *eaeA* gene were present in the food samples but at levels below the detection limit at t=0.

By the culture-dependent approach employed, 54 STEC strains were identified, originating from 32 food samples (31.7 % of the total). SMAC medium is commonly employed for the differentiation and identification of *E. coli* O157:H7, which is not able to ferment sorbitol and develops colorless colonies. For the purpose of this study, both colorless (presumptive *E. coli* O157:H7) and pink-red colonies were randomly picked up. Comparing the results presented in Tables 3 and 5, a clear difference is seen in the meat samples determined to contain STEC population(s) by qPCR (over 70 %) and by culturing and identification of isolates (27.4 %) at t=0. This difference for the dairy samples is less evident, 42 % positive by qPCR and 36 % positive by identification of isolates. These results confirm previous findings concerning the higher percent of prevalence of STEC when determined by PCR methods applied directly in food samples compared to culture based methods (Auvray et al., 2009; Madic et al., 2011; Pradel et al., 2000). Such deviation may partly be explained by the fact that isolates were randomly selected and that for most samples, a relatively low

STEC load was detected. However, for the meat samples, the difference between the two approaches is big and it could be associated with the physiology and overall state of the STEC populations in the specific samples. It has been suggested that such a discrepancy could be attributed to the stress to which bacteria are subjected during processing (Pradel et al., 2000). It should also be noted that a difficulty in STEC isolation has been previously reported (Auvray et al., 2007; Madic et al., 2011).

Taking into consideration the genotypes of the STEC strains, it can be said that the *stx*$_2$ gene was more often detected with respect to the *stx*$_1$, in fact several strains only carried the *stx*$_2$ gene (19 out of 54). This is of particular importance since it has been previously proposed that the Stx2 is the most common virulence factor associated with severe human disease (Pradel et a., 2008). No correlation could be observed between source of isolation (dairy or meat) and the presence of the *stx*$_2$ gene.

Limited information is available in the literature regarding the genotypes of STEC strains isolated from food sources. Pradel et al. (2008) compared the genotypes of STEC strains isolated from dairy products and from patients. Based on their findings the authors concluded that there was a statistical association between strains isolated from human disease and the presence of the *stx*$_2$ gene while the dairy STEC strains were characterized by the predominance of *stx*$_1$, with a limited number of strains carrying the *eaeA* gene. In a similar study conducted by Slanec et al. (2009), 75 STEC strains originating from foods, mainly meat but also some dairy products and water, were subjected to *stx* genotyping. In their study, they detected the *stx*$_1$ gene in 29 % of the strains and the *stx*$_2$ in 91 % of them. They also highlighted that a large number of the strains, although they contained the *stx*$_2$, they lacked any of the other virulence factors that were investigated in parallel. Also Beutin et al. (2007) detected the *stx*$_2$ gene in 80.8 % of STEC food isolates, the *stx*$_1$ gene in 40 % while the *eaeA* in only 5

% of them. In our study, the distribution of the virulence genes investigated partly confirmed these previous findings. Fifty five percent of total strains carried the *stx*$_1$ gene while 72.2 % encoded for the *stx*$_2$ gene. Furthermore, the low number of strains encoding the *eaeA* was confirmed. This finding may be an indication that these STEC strains, isolated from food sources, could present a low virulence potential. However, further investigation into other virulence genes is needed in order to provide more information in that regard. It should also be noted that among the 54 strains identified as STEC, 8 resulted to belong to the O157 serogroup, a characteristic that would allocate them among highly virulent strains for humans. These strains were isolated from 2 seasoned cheese samples and from 4 meat samples, 3 of them destined to be consumed without prior cooking. Strains belonging to other serogroups of food safety relevance, namely O25, O145, O103, O111, were not detected in this study. Finally, from the analysis performed on the strains, it could be seen that certain food samples (9 dairy and 7 meat) contained populations of STEC with different virulence genotypes. A similar finding has been presented by Slanec et al. (2009), that suggested the presence of multiple STEC populations in a food sample could result in infections caused by different pathogens present in a single source. Furthermore, in 9 samples, the genotypes detected before and after enrichment were different. We speculate that this is the result of competition of different STEC populations during enrichment.

The ERIC PCR, a method commonly used for fingerprinting enterobacterial genomes (Versalovic et al., 1991), was used in order to molecularly characterize the STEC strains. From the dendrogram obtained, it was seen that strains isolated from dairy samples grouped together, and formed relatively numerous clusters, while strains isolated from meat products were more heterogeneous and only formed two-strain

clusters or remained independent. Furthermore, a correlation was observed between the genotypes of strains and their grouping into clusters. Strains of the O157 serogroup, apart for two, were placed in different positions in the dendrogram, indicating that the diversity observed is not related to the serogroup of the strains.

This study highlights that when only culture dependent methods are applied in order to detect STEC in food samples, an underestimation of the real contamination load will probably be obtained. The high levels of STEC positive samples obtained are surprising. However, it has to be taken into consideration that until recently, data on STEC presence in foods were generated by culturing, isolation and identification. Apparently there is a need to reconsider the microbiological approaches used to determine the prevalence of STEC in food samples. Independently of the method employed, in this study the STEC prevalence resulted to be higher than what has been previously reported (Brooks et al., 2001; Doyle and Schoeni, 1987; Fantelli and Stephan, 2001; Pradel et al. 2000; Xia et al., 2010). This could be attributed to the different experimental approaches employed for the detection but also to the different types of foodstuffs considered. It should be underlined that the majority of foods tested in this study are considered safe since they are fermented and many of them are also characterized by long maturation/ripening steps during their production. The fact that about 42 % of the dairy and over 70 % of the meat samples resulted positive for STEC requires further investigation. The main issue raised concerns the actual virulence potential of these STEC populations detected.

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Figure legend

Figure 1. Dendrogram of similarity of the ERIC-PCR electrophoretic profiles obtained for the STEC strains isolated from dairy and meat products. The specific genotype determined for each strains is also shown. Strains determined to belong to serogroup O157 are marked with a black dot.

Table 1. Sequences of primers and probes and qPCR amplification conditions, used in this study.

Gene	Sequences	Conditions[a]
rpoB	rpoB_f: GTATGTCCAATCGAAACCCCT rpoB_r: GGTAGTGAATTTCGTCAGTTACA rpoB_probe: FAM-TCCGTATCGTAAAGTGACTGACGGTGTTGTA-TAMRA	50 cycles: 95 °C for 30 seconds 58 °C for 30 seconds 72 °C for 30 seconds
stx_1[b]	Ec_stx1_f: TGTCATTCGCTCTGCAATAGGTA Ec_stx1_r: CCCTCTGACATCAACTGC stx1_probe: HEX-CTGATGATTGATAGTGGC-TAMRA	40 cycles: 95 °C for 30 seconds 56 °C for 30 seconds 72 °C for 30 seconds
stx_2[b]	Ec_stx2_f: GTGGTAATACAATGACCAGAG Ec_stx2_r: ATCGTATACACAGGAGCAGT stx2_probe: FAM-CACAGCAGAAGCCTTACGC-TAMRA	40 cycles: 95 °C for 30 seconds 50 °C for 30 seconds 72 °C for 30 seconds
eaeA	Ec_eae_f: ATGTTGGGCTATAACGTCTTCAT Ec_eae _r: GTATGACTCATGCCAGCCGCTC eae _probe: FAM-GAGACTATTTCAAAAGTAGCGTTAACGGC-TAMRA	40 cycles: 95 °C for 30 seconds 50 °C for 30 seconds 72 °C for 30 seconds

[a]All cycles were preceded by an initial denaturation at 95 °C for 10 minutes. [b]Primers and probes for the stx_1 and stx_2 genes targeted the α-subunit and their specificity against the different variants was tested *in silico* using the Amplify 3.1.4 software (Bill Engels, 2005, University of Wisconsin).

Table 2. Efficiency and R^2 (correlation coefficient) for each gene in the different food matrices

Gene	**Food Matrix**	**Efficiency (%)**	**R^2**
rpoB	Fresh meat	91.2	0.992
	Soft cheese	91.2	0.997
stx$_1$	Fresh meat	91.4	0.984
	Soft cheese	97.2	0.988
stx$_2$	Fresh meat	84.7	0.988
	Soft cheese	90.9	0.970
eaeA	Fresh meat	62.2	0.930
	Soft cheese	54.6	0.855

Table 3. Prevalence of total *E. coli* and STEC as determined by culture independent method (qPCR at t=0 and t=24h). The percent of positive samples is shown in parenthesis and it was calculated separately for dairy and meat samples as well as for the total of samples.

		E. coli positive (*rpoB* gene amplification)	STEC positive (amplification of one or more of virulence genes)	*stx*$_1$ gene amplification	*stx*$_2$ gene amplification	*eaeA* gene amplification
Dairy products (50 samples)	t=0	ND[a]	21 (42%)	20 (40 %)	8 (16 %)	3 (6 %)
	t=24h	37 (74 %)	21 (42 %)	17 (34 %)	11 (22 %)	6 (12 %)
Meat products (51 samples)	t=0	ND	36 (70.6 %)	36 (70.6 %)	2 (3.9 %)	1 (2 %)
	t=24h	47 (92.1 %)	37 (72.5 %)	37 (72.5 %)	3 (5.9 %)	6 (11.8 %)
Total (101 samples)	t=0	ND	57 (56.4 %)	56 (55.4 %)	10 (9.9 %)	4 (4 %)
	t=24h	84 (83.2 %)	58 (57.4 %)	54 (53.5 %)	14 (13.9 %)	12 (11.9 %)

[a]ND: not determined

Table 4. Quantification of STEC positive samples as determined by application of the qPCR protocols directly in the food samples

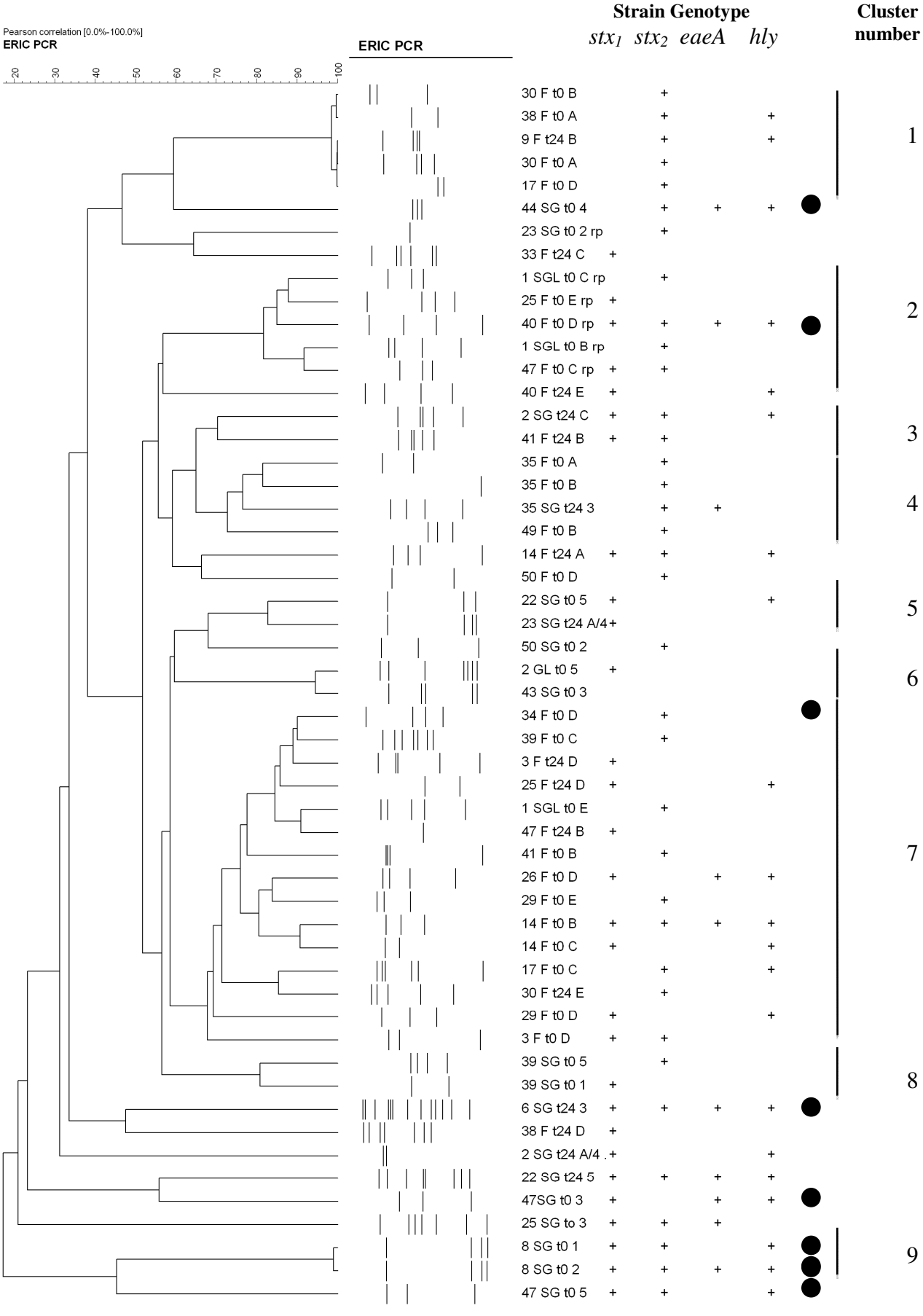
Sample code	Type of sample	Quantification of STEC population(s) by qPCR (cfu/g)		
		stx$_1$ quantification	*stx*$_2$ quantification	*eaeA* quantification
2SG	Fermented sausage	$2.86X10^2$	$1.10X10^3$	NA[a]
3F	Seasoned cheese	$8.51X10^2$	$3.11X10^3$	NA
9F	Seasoned cheese	$4.94X10^3$	$4.74X10^3$	$9.73X10^3$
14F	Fresh cheese	$1.48X10^2$	$9.04X10^2$	NA
17F	Seasoned cheese	$4.05X10^2$	$1.55X10^2$	NA
26F	Fresh cheese	$2.52X10^4$	$4.83X10^2$	NA
49F	Seasoned cheese	$4.19X10^2$	$1.45X10^2$	NA

[a]Not applicable: samples were negative or below the quantification limit of the method and thus quantification was not possible

Table 5. Distribution of STEC genotypes as determined by multiplex PCR (Paton and Paton, 1998b), Strains were picked up from SMAC plates before and after an overnight enrichment of food samples in BHI broth (see materials and methods for more details). Strains that were assigned to the O157 serogroup by PCR amplification are highlighted.

Sample Code	Number of strains	Genotype of strain(s) isolated before enrichment	Number of strains	Genotype of strain(s) isolated after enrichment
Dairy products				
3F	1	$stx_1^+ stx_2^+$	1	stx_1^+
9F	0		1	$stx_2^+ hly^+$
14F	2	$stx_1^+ stx_2^+ eaeA^+ hly^+$ $stx_1^+ hly^+$	1	$stx_1^+ stx_2^+ hly^+$
17F	2	$stx_2^+ hly^+$ stx_2^+	0	
25F	1	stx_1^+	1	$stx_1^+ hly^+$
26F	1	$stx_1^+ eaeA^+ hly^+$	0	
29F	2	$stx_1^+ hly^+$ stx_2^+	0	
30F	2	stx_2^+ stx_2^+	1	stx_2^+
33F	0		1	stx_1^+
34F	1	stx_2^+	0	
35F	2	stx_2^+ stx_2^+	0	
38F	1	$stx_2^+ hly^+$	1	stx_1^+
39F	1	stx_2^+	0	
40F	1	$stx_1^+ stx_2^+ eaeA^+ hly^+$	1	$stx_1^+ hly^+$
41F	1	stx_2^+	1	$stx_1^+ stx_2^+$
47F	1	$stx_1^+ stx_2^+$	1	stx_1^+
49F	1	stx_2^+	0	
50F	1	stx_2^+	0	
Number of dairy samples with STEC presence: 18 (36%)	**Total: 21 strains**		**Total: 10 strains**	
Meat products				
1SGL	3	stx_2^+ stx_2^+ stx_2^+	0	
2GL	1	stx_1^+	0	
2SG	0		2	$stx_1^+ hly^+$ $stx_1^+ stx_2^+ hly^+$
6SGL	0		1	$stx_1^+ stx_2^+ eaeA^+ hly^+$
8SGL	2	$stx_1^+ stx_2^+ hly^+$ $stx_1^+ stx_2^+ eaeA^+ hly^+$	0	
22SGL	1	$stx_1^+ hly^+$	1	$stx_1^+ stx_2^+ eaeA^+ hly^+$
23SGL	1	stx_2^+	1	stx_1^+
25SGL	2	$stx_1^+ stx_2^+ eaeA^+ hly^+$ $stx_1^+ stx_2^+ eaeA^+$	0	
35SGL	0		1	$stx_2^+ eaeA^+$
39SGL	2	stx_1^+ stx_2^+	0	
43SGL	1	stx_2^+	0	
44SGL	1	$stx_2^+ eaeA^+ hly^+$	0	
47SGL	2	$stx_1^+ eaeA^+ hly^+$ $stx_1^+ stx_2^+ hly^+$	0	
50SGL	1	stx_2^+	0	
Number of meat samples with STEC presence: 14 (27.4 %)	**Total: 17 strains**		**Total: 6 strains**	
Total number samples with STEC presence: 32 (31.7 %)	**Total: 38 strains**		**Total: 16 strains**	

Figure 1


Pearson correlation [0.0%-100.0%]
ERIC PCR
ERIC PCR
20
30
40
50
60
70
80
90
100
Strain Genotype
stx1 stx2 eaeA hly
Cluster number
30 F t0 B + 1
38 F t0 A + +
9 F t24 B + +
30 F t0 A +
17 F t0 D +
44 SG t0 4 + + +
23 SG t0 2 rp +
33 F t24 C +
1 SGL t0 C rp + 2
25 F t0 E rp +
40 F t0 D rp + + + +
1 SGL t0 B rp +
47 F t0 C rp + +
40 F t24 E + +
2 SG t24 C + + + 3
41 F t24 B + +
35 F t0 A +
35 F t0 B + 4
35 SG t24 3 + +
49 F t0 B +
14 F t24 A + + +
50 F t0 D +
22 SG t0 5 + + 5
23 SG t24 A/4 +
50 SG t0 2 +
2 GL t0 5 + 6
43 SG t0 3
34 F t0 D +
39 F t0 C +
3 F t24 D +
25 F t24 D + +
1 SGL t0 E +
47 F t24 B +
41 F t0 B + 7
26 F t0 D + + +
29 F t0 E +
14 F t0 B + + + +
14 F t0 C + +
17 F t0 C + +
30 F t24 E +
29 F t0 D + +
3 F t0 D + +
39 SG t0 5 + 8
39 SG t0 1 +
6 SG t24 3 + + + +
38 F t24 D +
2 SG t24 A/4 . + +
22 SG t24 5 + + + +
47SG t0 3 + + +
25 SG to 3 + + +
8 SG t0 1 + + + 9
8 SG t0 2 + + + +
47 SG t0 5 + + +